ISOENZYMES OF AN ACYL TRANSFERASE FROM RABBIT MAMMARY GLAND: EVIDENCE FROM BIPHASIC SUBSTRATE SATURATION KINETICS

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1. Introduction

Biphasic substrate saturation curves (V/S curves) observed for enzymes acting on water soluble substrates have been attributed to negative co-operativity [1]. With lipid substrates, the biphasic phenomenon results from assaying at concentrations above and below the substrate critical micelle concentration (cmc) and the enzyme is assumed to interact with both monomeric and micellar forms of the substrate [2,3]. Such atypical Michaelis curves were obtained with a microsomal preparation of acyl-CoA: lysophosphatidate acyltransferase (LPAT) from the mammary gland of rabbits in late pregnancy and early lactation when lysophosphatidic acid (LPA) was the variable substrate. However, in early pregnancy the enzyme was active only with monomers and micellar substrates were inhibitory. Hence, the ability of the mammary cell to function with micelles appears to be acquired during cellular differentiation in mid-pregnancy and is independent of its ability to utilize monodisperse substrate forms. In this report we present evidence for the presence of two LPAT isoenzymes in the microsomes of rabbit mammary gland. One of the isoenzymes appears to be monomer and the other micelle specific.

2. Materials and methods

Mammary tissue was excised from pregnant and lactating rabbits (Dutch Belted variety), homogenized in 0.25 M sucrose and differentially centrifuged to obtain a microsomal preparation [4]. The microsomal suspension in 0.25 M sucrose was then sonicated (Cole-Palmer Ultrasonic Cleaner, Model 8845-3) for 1 min. The assay method is essentially that of Lands and Hart [5] with the exception that 4,4′-dithiodipyridine (Aldrithiol-4) was the sulphydryl-binding reagent.

Prior to assay the enzyme was preincubated for four min in the reaction medium without substrates. After the rate of palmityl-CoA hydrolysis was measured (1.5 min) monopalmityl-*sn*-glycerol-3-phosphate (lysophosphatidate) was added and the absorbance change at 324 nm recorded continuously for 2–4 min in quartz cells (1 cm light path) with a 10 mV Hitachi Perkin-Elmer Model 165 recorder attached to a Perkin-Elmer Model 365 spectrophotometer. Correction for hydrolase activity was made in all cases. The molar absorbtivity 17 700 M^{-1}, cm^{-1} was used to calculate the amount of 4-thiopyridone released in the reaction. Protein was measured using a modification of the Lowry method [6] in that the reaction mixture was incubated for 10 min at 50°C after addition of reagent C and again for 10 min at 50°C upon addition of reagent E.

3. Results

Michaelis-Menten kinetics were obtained when LPAT was assayed in microsomes of mammary tissue from rabbits in early pregnancy (fig.1a). Above 50 μM LPA severe substrate inhibition was observed. Under conditions of assay 50 μM LPA of 70 μM total surfactant (i.e. palmityl-CoA plus LPA) is assumed to correspond with the cmc of this surfactant pair [7–9] and the enzyme is regarded as being specific for monomeric substrate molecules [10]. After the

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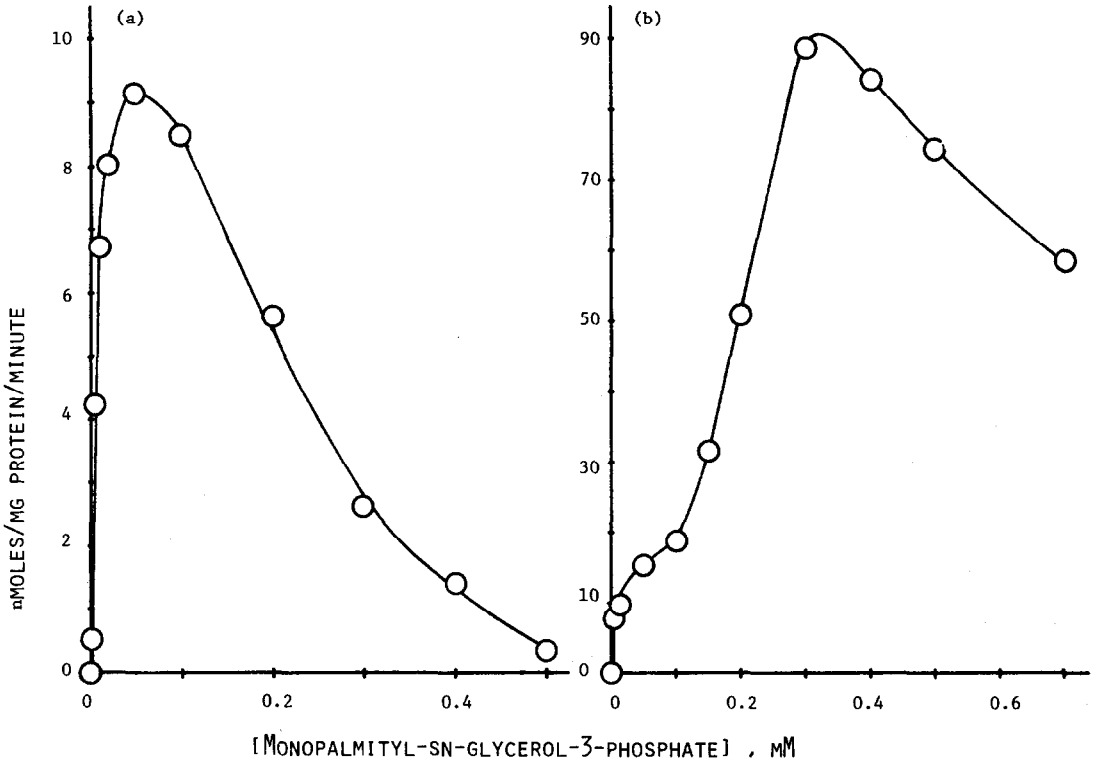

Fig.1. Acylation of monopalmityl-*sn*-glycerol-3-phosphate by rabbit mammary gland microsomes in a) early pregnancy and b) lactation. Reaction mixtures contained Tris–HCl buffer, 70 mM, pH 7.4; 4,4'-dithiodipyridine (Aldrich), 0.3 mM; palmityl-CoA (P-L Biochemicals), 18 μM; microsomal protein, 0.23 mg/ml; and monopalmityl-*sn*-glycerol-3-phosphate (Serdary) as indicated. Specific activity is expressed as nmoles palmitate transesterified/mg protein/min.

second week of pregnancy, however, a second peak appeared in the V/S curve above the apparent cmc (fig.1b) and this biphasic pattern persisted into lactation. The appearance of this second peak suggests the presence of two LPAT isoenzymes in functional rabbit mammary gland from mid-pregnancy onwards. One isoenzyme appears to require soluble and the other micellar substrates. These multiple forms of LPAT are referred to as isoenzymes α and β respectively.

Additional properties of the two LPAT isoenzymes were examined to corroborate these findings. Enzyme stability was compared by assaying at intervals over a 23 hr period at 27°C, using 40 μM and 400 μM LPA, the optimum acceptor concentration for isoenzymes α and β, respectively. The results (fig.2) are consistent with the presence of two LPAT activities.

At fixed levels of palmityl-CoA (18 μM) and LPA (40 μM) the dependence of reaction rate on protein concentration was observed to be biphasic (fig.3). These data reflect a change in the physical state of the substrates as the level of protein was increased and the appearance of two distinct LPAT activities in response to this change.

Assuming isoenzyme α alone is present in the early pregnancy preparations it is possible to graphically resolve the *V/S* curve for isoenzyme β from the biphasic *V/S* curve in fig.1b, using a procedure similar to that of Walaas et al. [11]. *V/S* data points for isoenzyme α (fig.1a) were normalized to those observed in the biphasic curve (fig.1b) and are shown as open circles in fig.4. Curve B (fig.4), obtained by subtracting normalized data points for isoenzyme α from those observed in the biphasic *V/S* curve, represents the resolved Michaelis curve for isoenzyme β and can be defined kinetically by the Hill equation

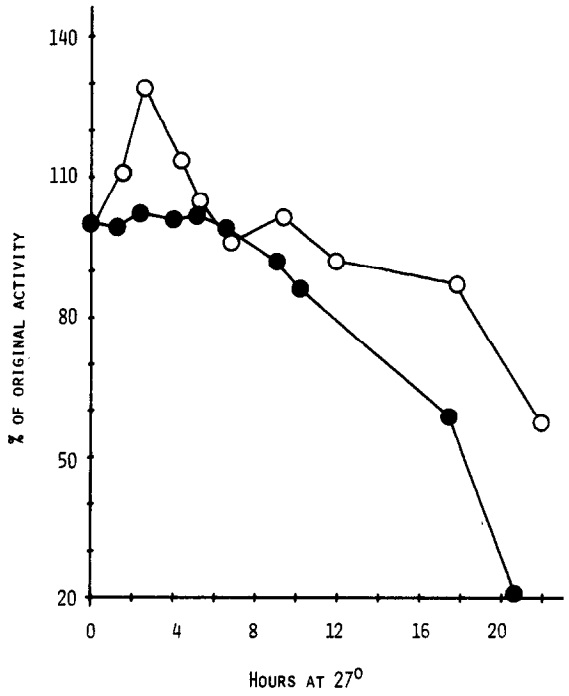

Fig.2. Stability of microsomal palmityl-CoA: monopalmityl-*sn*-glycerol-3-phosphate palmityltransferase activities from lactating rabbit mammary gland at 27°C. Reaction mixtures as specified in fig.1 with 40 μM (●–●) and 400 μM (○–○) monopalmityl-*sn*-glycerol-3-phosphate.

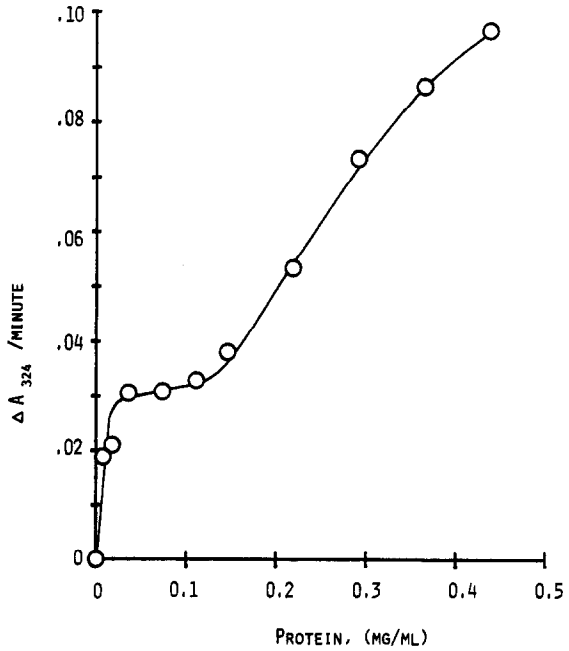

Fig.3. Dependence of palmityl-CoA: monopalmityl-*sn*-glycerol 3-phosphate palmityltransferase reaction rate on the concentration of enzyme from lactating rabbit mammary tissue. Reaction mixture as specified in fig.1 with 40 μM monopalmityl-*sn*-glycerol-3-phosphate.

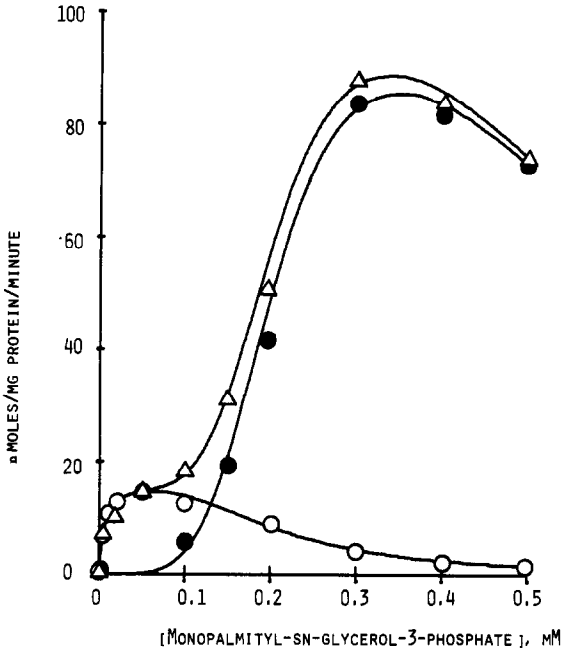

Fig.4. Resolution of the substrate saturation curve of isoenzyme-β (●–●), curve B) by point for point substration of normalized data points in fig.1a (○–○), curve A) from data points in fig.1b (△–△) curve C). Curves A, B and C were generated by computer using the rate equations presented in the text.

[12] incorporating a term to describe cooperative substrate inhibition:*

$$V_{o_\beta} = \frac{V_{max_\beta}(S)^n}{[(S)^n + K]\ [1 + (S)^{m''}/K_{s_\beta}]}$$

*V_o is the initial reaction velocity; S, the L-PA concentrations; V_{max}, the maximum reaction rate possible with a fixed amount of enzyme; n, the Hill coefficient; K, the Hill constant; m, the coefficient catering for co-operative substrate inhibition; K_s, the dissociation constant of the inactive enzyme–substrate complex and K_m, the Michaelis-Menten constant.

when V_{max_β} = 106 nmol/mg protein/min.; n = 4.5; $K = 2.5 \times 10^{-3}$ M; m'' = 3.2; $K_{s_\beta} = 4 \times 10^{-2}$ M; and V_{o_β} and S are varied.

A best fit to the normalized data points for isoenzyme α (curve A, fig.4) was obtained using the rate equation for cooperative substrate inhibition in a single substrate reaction,

$$V_{o_\alpha} = \frac{V_{max_\alpha} S}{[S + K_m]\ [1 + (S)^{m'}/K_{s\alpha}]}$$

when V_{max_α} = 16.8 nmol/mg protein/min; $K_m = 6.4 \times 10^{-6}$ M; $K_{s\alpha} = 6.8 \times 10^{-4}$ M; m' = 2.7 and V_{o_α} and S are varied. Under assay conditions, palmityl-CoA was at saturating concentrations and it is therefore valid to use the above rate equation. Curve C (fig.4) is then described by V_{ot}, the summation of $V_{o\alpha}$ and $V_{o\beta}$, as follows:

$$V_{ot} = \frac{V_{max_\alpha} S}{[S + K_m]\ [1 + (S)^{m'}/K_{s\alpha}]} + \frac{V_{max_\beta} (S)^n}{[(S)^n + K]\ [1 + (S)^{m''}/K_{s_\beta}}$$

The observed biphasic V/S curve can thus be resolved into two kinetically distinct components: a Michaelis-Menten and an allosteric type where co-operative substrate inhibition is common to both. It is not known at the present time whether allosteric activation is real or merely reflects an effect of phase change and a differential reaction rate of isoenzyme β toward monodisperse and aggregated substrates molecules. As a result of non-specific binding and micellarization of substrate molecules, the kinetic parameters presented above should be considered functions rather than constants.

4. Discussion

The evidence presented indicates that LPAT activity in differentiated rabbit mammary tissue is represented by two isoenzymic species. The isoenzymes differ with respect to the physical nature of the substrates with which they interact. When substrates are in the monodisperse form, LPAT acts on molecular species of less that 1000 daltons. Above the cmc, substrate molecules are presented as part of a micelle, the molecular weight of which may approach or exceed that of the enzyme itself. At pH 7.4, mixed micelles of palmityl-CoA and LPA may be considered as macromolecular polyanions and it is with these structures that the enzyme must interact. The existance therefore of separate LPAT isoenzymes for utilizing substrates in such dissimilar physical states is plausible.

That this monomer-micelle specificity resides with two discrete proteins may be deduced from the observed differences in stability of the two isoenzymes at 27°C and from the fact that although isoenzyme α was present throughout pregnancy and lactation, β activity was not evident until mid-pregnancy.

Considering the difference in catalytic capacity of the two forms of LPAT in lactation (fig.1b) and the fact that isoenzyme β does not appear until mid-pregnancy, one may speculate on the physiological significance of this isoenzyme pair apart from the assigned monomer-micelle specificity. Phospholipid turnover occurs in resting undifferentiated mammary cells and a low level of LPAT is required. In the differentiated cell triglycerides for secretion are synthesized at a rapid rate (lactating rabbit mammary gland secretes approx. 22 g fat/day [13]). Hence, it is conceivable that LPAT isoenzymes α and β reflect the normal consitutive activity and the much higher activity required in lactation, respectively. The two isoenzymes, therefore may be involved in the synthesis of phosphatidic acid for two discrete diglyceride pools, one small pool for the synthesis of phosphoglycerides and a second larger pool for convertion to secretory triglycerides. DeKruyff et al. [14] have suggested that such pools exist in rat liver cells. In this context, it seems reasonable to consider the possibility of isoenzymes α and β being interconvertible forms of the one enzyme, where the equilibrium between α and β forms is regulated by certain physiological parameters of pregnancy and lactation.

Acknowledgements

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References

[1] Levitzki, A. and Koshland, D. E. (1969) Proc. Natl. Acad. Sci. U.S. 62, 1121–1128.
[2] Lipovac, V., Bigalli, G. and Rosenberg, A. (1971) J. Biol. Chem. 246, 7642–7648.
[3] Viljoen, C. C., Schabort, J. C. and Botes, D. P. (1974) Biochim. Biophys. Acta 36, 156–165.
[4] McDonald, T. M. and Kinsella, J. E. (1973) Arch. Biochem. Biophys. 156, 223–231.
[5] Lands, W. E. M. and Hart, P. (1965) J. Biol. Chem. 240, 1905–1911.
[6] Lowry, H. O., Rosebrough, N. J., Farr, A. L. and Randal, R. J. (1951) J. Biol. Chem. 193, 265–275.
[7] Barden, R. E. and Cleland, W. W. (1969) J. Biol. Chem. 244, 3677–3684.
[8] Okuyama, H. and Lands, W. E. M. (1972) J. Biol. Chem. 247, 1414–1423.
[9] Yamashita, S., Kosaka, K. and Numa, S. (1973) Eur. J. Biochem. 38, 25–31.
[10] Gatt, S., Barenholz, Y., Borkovski-Kubiler, I. and Leibovitz-Ben Gershon, Z. (1972) Adv. Exp. Med. Biol. 19, 237–256.
[11] Walaas, O., Walaas, E. and Osaki, S. (1967) in: FEBS Symposium: Control of glycogen metabolism (Whelan, W. J., ed), pp 139–152, Academic Press, London and New York.
[12] Wold, F. (1971) Macromolecules: Structure and Function, pp. 26–39, Prentice-Hall, Inc., New Jersey.
[13] Linzell, J. L. (1972) Dairy Sci. Abstr. 34, 351–360.
[14] DeKruyff, B., VanGolde, L. M. and van Deenan, L. L. M. (1970) Biochim. Biophys. Acta 210, 425–435.